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Resistance to *Pythium debaryanum* in *Zea mays* Seedlings[1]

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Seedling resistance to *Pythium* was studied by placing maize kernels on or under a culture of *Pythium* growing on a semisynthetic medium. The main criteria were living plumules and roots after 14-days incubation at 12 C and 6 days at 23-27 C. Greater mortality occurred when inoculum was above the kernels than below. Surface-sterilization of kernels before exposure to *Pythium* increased seedling mortality, but moisture content of kernels at the start of exposure had only a slight effect on survival at 12 C. Maximum mortality occurred when the cold period was more than 8 days. Survival was greater after a short cold period of 1-2 days than if the kernels were not exposed to any cold-incubation period. Varying the length of the cold incubation introduces a genetic-incubation interaction. The critical period in exposure to *Pythium* at low temperatures was between 48 and 96 hours of exposure, by which time the fungus had become established in the kernels, and surface sterilization would not free them from infection. All kernels, from susceptible or resistant genotypes, were invaded in 4 days. A daily alternating incubation temperature (10-25 C) was highly variable in effect on survival. The expression of resistance was maximum in the plumule and scutellar node, which, if they survived the initial exposure to *Pythium*, showed near immunity. The radicle and seminal roots were never longer than 1 cm if exposed to *Pythium*, except in seedlings having a resistant plumule.
INDEX DESCRIPTORS: "cold" test, seed infection, seedling blight, root rot.

Major resistance to *Pythium debaryanum* Hesse is manifested by the embryo of *Zea mays* L. (4). Specific tissues, pericarp, endosperm, and scutellum, are important in the expression of resistance; the pericarp protects the germinating embryo from pathogens (6). Characteristics of the pericarp possibly account for the commonly observed maternal effect in tests of reciprocal crosses. Hooker and Dickson (4) found that all embryos are susceptible in early stages of germination and that resistant lines are the ones in which embryos develop resistance faster than embryos of susceptible lines. Resistance was expressed by the excised embryo and was not dependent on the protection of an intact pericarp.

Pythium is an important pathogen of maize (2, 5, 7, 9, 10), but the methods for studying seedling diseases are less than adequate.

This study was concerned with the development of a test for observing the expression of resistance without the interference of extraneous organisms, the identification of factors causing variability of response in the "cold" test, and the evaluation of the responses of dent-maize seedlings.

MATERIALS AND METHODS

The culture of *Pythium debaryanum* was isolated originally by P. Hoppe, University of Wisconsin, from rotted maize kernels. Stock cultures were maintained on potato-dextrose agar, but inoculum was produced on a multipurpose basal medium suitable for growth of maize seedlings and fungal mycelium if a carbon source is added. The basal medium contained NH_4NO_3, 0.32g; $CaCl_2$, 0.285g; KCl, 0.285g; KH_2PO_4, 0.285g; $MgSO_4 \cdot 7H_2O$, 0.427g; biotin, 5 μg; and thiamine, 100 μg (all per liter). Trace amounts of $MnCl_2 \cdot 4H_2O$, H_3PO_3, $ZnSO_4$, and MnO_3 were added to the dilute solution. The major minerals were dissolved separately in water and maintained as 100-fold stock solutions. Glucose (5 g/liter) or cellulose (9-cm diam sheet of filter paper/10 ml mineral solution) was the carbon source. Glucose, mineral solution, and agar (17 g/liter) were sterilized, combined, poured into sterile 9-cm diam Petri dishes seeded with a *Pythium* inoculum disc (0.5-cm diam), and incubated 4 days at 23-27 C. If cellulose was the carbon source, *Pythium* was grown in test tubes containing 10 ml of basal medium and 1 sheet of folded paper. The sheet of *Pythium*-bearing paper was spread on top of the test kernels and agar.

Maize kernels in small bottles were flooded for less than 1 min with 1.3% sodium hypochlorite solution. The solution was decanted, and the bottles were plugged with nonabsorbent cotton and placed in a forced-air drying oven 4 days at 35 C. The kernels were then arranged evenly, without regard to embryo orientation, on top of the 4-day-old *Pythium* cultures (regular test) or directly on the glucose-mineral agar and covered with the *Pythium*-bearing filter paper (covered test).

The Petri dishes were placed in covered plastic boxes, which were placed in insulated thermostatically controlled unlit incubators. Incubation temperatures were measured with a mercury thermometer graduated in 0.1 C divisions. The cold incubation temperature was 12 ± 0.1 C and, unless specified otherwise, incubation lasted 14 days; then the tests were returned to room temperature (23-27 C).

The number of living seedlings was recorded after 6 days at room temperature. Seedlings that were alive and green at 6 days were counted as living. Embryos were classed as dead that did not emerge, had a translucent water-soaked appearance, or were necrotic. Seedlings were considered to have a root if the primary radicle was longer than 1 cm. Unless specified otherwise, a test (1 replicate) consisted of 25 kernels from one ear in a Petri dish containing medium and inoculum.

All maize kernels were from hand-shelled ears grown at Ames, Iowa. Any ear showing symptoms of ear or cob rot was discarded at harvest, and ears that did not produce 95% living seedlings in uninoculated tests were eliminated.

Inoculum placement

Three arrangements of inoculum placement in relation to kernels were tested. Arrangement A was obtained by placing the 25 kernels in a sterile Petri dish and pouring 20 ml of mineral agar (55 C) around the kernels. After the agar had solidified and cooled, *P. debaryanum* growing on a 9-cm diam sheet of paper was placed over the kernels. In arrangement B, the mineral agar was poured and allowed to cool before the kernels were placed on the surface of the solidified medium. The inoculum was added as in A. Arrangement C was similar to B, except that the inoculum was placed on the medium before the kernels were added. Tests of the effect of inoculum placement were repeated using three types of inoculum carriers — i) disc of filter paper saturated with mineral medium, ii) disc of glucose-mineral agar, and iii) autoclaved maize kernels (one infested kernel was placed carefully on each test kernel).

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Inter-kernel influence on survival

Four populations (3, 6, 12, and 24 kernels/plate) of hybrids U.S. 13 and B14 × Wf9 were tested by the covered (arrangement B) method in 5 replications. A susceptible yellow kernel hybrid (Wf9 × B14) and a resistant purple kernel hybrid (127 × 129) were mixed in 6 proportions of yellow:purple of 25:0, 24:1, 20:5, 15:10, 5:20, and 0:25.

RESULTS

Radicles emerged 1 or 2 days before the plumules from uninoculated kernels, whereas in tests with inoculated kernels, radicle emergence was retarded or did not occur. The average length of radicles from inoculated kernels after the cold incubation plus 6 days at 25 C was less than 1 cm; radicles of uninoculated kernels similarly incubated usually exceeded 10 cm (range 6-15 cm). About 90% of the radicles of inoculated kernels were killed before emergence. A few radicles emerged and occasionally produced secondary roots, but the root tips were killed. Seminal roots were common in surviving embryos, but eventually the tips became necrotic. Roots infected by *Pythium* developed localized necrotic lesions that occasionally girdled the root.

In contrast to roots, the plumules did not exhibit local lesions. If the plumule was not killed at emergence, it continued to grow; and, if the seedling was removed from the *Pythium* culture, it would produce new roots and survive when transplanted to soil. Uninoculated kernels germinated at the rate of 96% or better. Killed embryos frequently had profuse mycelial growth emanating from the embryo part of the kernel.

Orientation of the embryo in relation to the inoculum did not greatly affect survival. Resistant embryos emerged whether facing down and contacting mycelium of *Pythium* or facing up and not in contact with mycelium at the start of incubation.

Inoculum placement

The survival of seedlings exposed to *Pythium debaryanum* was affected by the position of inoculum in relation to the kernels (Table 1).

Table 1. *Survival (%) of maize embryos of four inbred lines inoculated with* P. debaryanum *in three arrangements and incubated 14 days at 12 C.*

Cultivar	Number of ears tested[a]	(A) Inoculum on top of kernels embedded in medium	(B) Inoculum on top of kernels on medium	(C) Kernels on top of inoculum on medium
		%	%	%
C103[b]	8	1.5	6.0	23.5
Hy	14	4.3	14.0	38.3
Wf9	11	0.0	2.5	4.7
B2	8	0.0	1.5	9.0
Survival means[c]		1.8	6.9	20.7

[a] Twenty-five seed per test.

[b] The F-ratio for mean squares between cultivars was 18.42, significant at 1% level.

[c] The F-ratio for mean squares between arrangements was 30.66, significant at 1% level.

Table 2. *Survival (%) of maize embryos in covered tests of varying proportions of a resistant purple-kernel hybrid (127 × 129) to a susceptible yellow-kernel hybrid (Wf9 × B14).*

Ratio of yellow:purple	Survival of indicated kernels[a]	
	yellow	purple
	%	%
25:0	25	—
24:1	29	60
20:5	41	92
15:10	17	74
5:20	32	81
0:25	—	84

[a] Mean of two replications.

The medium formulation, sterilization procedure, and length and temperature of incubation were the same for the different arrangements. The effect of inoculum placement was retested using three types of inoculum carriers — i) disc of filter paper saturated with mineral medium, ii) disc of glucose-mineral agar, and iii) autoclaved maize kernels. These gave 6.3, 1.3, and 8.8% seedling survival, respectively. The placement of sterile paper on top of kernels with *Pythium* beneath did not duplicate the increase associated with *Pythium* and paper on top. Seven inbred lines and 3 hybrids (2 sets each) were exposed to *Pythium* in regular tests by covering one set with sterile saturated filter paper; the other set was not covered, and seedling survival was 37.2 and 44.0%, respectively. More than 95% of the uninoculated kernels germinated.

Inter-kernel influence on survival

Susceptible and resistant embryos did not interact or influence survival of each other when confined in a Petri dish. The survival of embryos at the populations of 3, 6, 12, and 24 per dish was 13.3, 3.3, 6.7, and 3.8%, respectively. In the experiment with mixed genotypes, the survival of the susceptible yellow-dent-kernel hybrid (Wf9 × B14) was not significantly affected by mixing in different proportions with the resistant purple-dent hybrid (127 × 129); likewise, the survival of the resistant kernels was not affected by the presence of the susceptible kernels (Table 2).

Intra- and inter-ear variations in embryo survival

Six subsamples of 25 kernels from one ear from each of 4 lines were compared in the regular *Pythium* test. The range and average number of survivors was: for Wf9 0-0, 0; Hy 1-8, 3; B37 0-1, 0.3; and for I205 2-8, 5.2 embryos. The error mean square of the transformed numbers ($\sqrt{\times + 0.5}$) was 0.183. Experimental and ear variation were compared further in 10 inbred lines by testing 2 samples of 5 ears each by the regular test (Table 3). The mean squares of survival were greater between ears than within ears in 7 of the 10 lines; and, in the combined analysis of variance, the differences in survival between ears were significant at the 1% level. Differences between ears due to variation within inbred lines has been noted (3). Clearly, it was more efficient to sample more ears and seed sources than to run several tests of one or few ears. The results of other resistance tests (unpublished data) verified the uniformity that can be expected in the regular *Pythium* test, provided that kernels are not physically damaged or infected with the ear pathogens *Diploida zeae*, *Fusarium graminearum*, and *Nigrospora oryzae*.

Moisture factors of the Pythium test

The effect of surface sterilization on embryo survival in the regular

Table 3. *Survival (%) of embryos in two replications (I, II) per ear of five ears each of ten inbred maize lines exposed to* P. debaryanum *at 12 C for 14 days (regular test).*

Cultivar	Ear Number[a] 1		2		3		4		5		Mean
	I	II	I	II	I	II	I	II	I	II	
	%	%	%	%	%	%	%	%	%	%	%
Wf9	0	0	12	0	0	0	0	0	0	0	1
Hy	0	0	12	0	28	16	20	8	20	48	15
B37	16	8	24	16	0	4	4	4	24	4	10
I205	40	16	16	24	12	0	4	24	8	8	15
C103	12	4	0	8	28	44	24	52	16	0	19
B14	32	28	48	64	28	24	36	16	44	28	35
B2	0	0	0	0	0	0	0	0	4	0	0
Oh5la	36	24	16	40	32	52	28	24	16	20	29
M14	0	0	4	0	0	0	0	12	4	0	2
CI90a	0	0	0	12	0	0	0	0	0	0	1

[a] The F ratio for mean square between ears within lines was 2.52, significant at 1% level.

test was studied in a hybrid W61 and a synthetic population (S_1). The development of plumules from nonsurface-sterilized kernels was about twice that of surface-sterilized kernels. Evidently the protective surface flora of nonsterilized kernels is effective in vitro, as in soil (1). Some kernels were surface-sterilized by immersion in a freshly prepared solution of calcium hypochlorite (4 g/1), and no toxicity of residual chlorite to *Pythium* was observed. Alternate wetting and drying of kernels did not affect germination.

Moisture content of kernels of Wf9 and B14 at the start of exposure to *Pythium* was adjusted by imbibing kernels on sterile, moistened blotting paper at 1 C for 0, 48, and 96 hours. Moisture content of subsamples determined gravimetrically was 9-10, 25-26, and 29-30% for 0, 48, and 96 hours of imbibition, respectively. Samples (25 kernels) from each of four ears each of Wf9 and B14 that had been imbibed at 1 C were exposed to *P. debaryanum* by the covered method (arrangement B). Inbred Wf9 produced no survivors at any level of imbibition, and inbred B14 produced 17, 24, and 25% survivors with 0, 48, and 96 hours of imbibition, respectively.

Invasion time at low temperatures

Kernels of Wf9 and Hy were placed on mineral agar and covered with a culture of *Pythium* on a glucose-mineral agar 9-cm disc inverted on the kernels (pancake style) and incubated at 12 C. Periodically, kernels were removed from the test plates, washed with 1.3% sodium hypochlorite, and placed on sterile mineral agar at 26 C. Living seedlings and presence of white mycelium were recorded 6 days after transfer to 26 C. The length of exposure necessary for killing was not related to the final mortality (Table 4). Kernels exposed for 24 hours showed no evidence of invasion; after 48 hours, about 25% of the kernels had been invaded, but killing was more variable than with exposure for 8 or 14 days.

In another experiment, the time of exposure at 12 C was varied, but the kernels were not removed from the *Pythium* culture when transferred to 26 C. One hybrid (U.S. 13) and two inbred-lines, B14 and Wf9, were treated by the covered test (arrangement B) at 12 C for 2-24 days. Survival at 2, 4, and 6 days was 85, 40, and 30% of U.S. 13; 45, 5, and 0% of Wf9; and 50, 10, and 10% of B14 kernels, respectively. Wf9 and B14 did not survive incubations longer than 6 days. Average survival of U.S. 13 was 13% in incubations longer than 6 days. The experiment was repeated by using hybrids U.S. 13 and Wf9 × B14 and inbred lines B14 and Wf9 and more incubation periods at 12 C (Table 5). Incubation at 25 C without a cold incubation was more detrimental to embryo survival than a short period (2 days) of exposure at 12 C. Differences in survival at the various periods of incubation were significant at the 5% level.

The effect of *Pythium* on embryo survival (regular test) was erratic at alternating temperatures (10 C for 20 hours and 25 C for 4 hours) in a growth chamber with 1 hour required to equilibrate at each cycle change. Plumules started emerging at 8 days and continued until 20 days. The percentage survival of three inbred lines in four tests each averaged 54% for C103, 44% for Hy, and 59% for Wf9. The survival of Wf9 was the most variable, ranging from 36 to 84%. Survival was greater and more variable at alternating temperatures than at constant temperatures.

Classification of responses

The results of tests of > 5000 ears of segregating populations were summarized according to the possible combinations of reactions of the plumules and radicles. The segregating populations were F_2 and F_3 ears of nine single crosses and several generations of self pollinated ears of a synthetic population made by combining eight inbred lines. The possible classes of embryos considered were:

Table 4. *Survival (%) of embryos of two maize lines after indicated days of exposure to* P. debaryanum *at 12 C (arrangement B). Embryos were counted after removal from culture and incubation of 6 days at 25 C.*

Cultivar	Days of exposure to *Pythium*[a] 1	2	4	8	14
	%	%	%	%	%
Wf9	99	95	79	0	0
Hy	97	95	18	28	1

[a] Three replications per treatment.

Table 5. *Survival (%) of embryos of four maize cultivars exposed to* Pythium *at 12 C for nine periods of incubation (covered test).*

Cultivar	Days of incubation at 12 C[a]								
	0	0.5	1	1.5	2	3	4	8	14
	%	%	%	%	%	%	%	%	%
U.S. 13	30	20	40	60	55	30	20	5	0
Wf9 × B14	10	10	20	30	35	20	10	0	0
B14	5	10	10	10	0	10	10	0	0
Wf9	0	0	0	5	0	0	0	10	0
Mean survival[b]	11	10	17	26	22	15	10	4	0

[a] Ten kernels per plate and two places per treatment.

[b] The F ratio of the mean squares between days was 4.78, significant at the 1% level.

I. Susceptible plumule and susceptible radicle
II. Resistant plumule and susceptible radicle
III. Resistant plumule and resistant radicle
IV. Susceptible plumule and resistant radicle

Class I reaction was the most commonly observed combination; ClassII was the second most frequent combination of response. Class IV was looked for, but never observed in any seedlings from more than 5000 ears studied. The frequencies of Classes I-III depended on the cultivars being tested, but the ratio of occurrences of Class II to Class III was relatively constant at about 4:1.

DISCUSSION

An *in vitro* test of survival of embryos exposed to a pathogen has several advantages. The growth of the pathogen is visible, and juxtaposition of inoculum and kernel can be noted. Internal seed inhabitants that might interfere with a soil "cold" test are detectable. The appearance and location of necrosis can be noted without sacrificing the seedling.

The test is reasonably reproducible, and differences between tests within an ear are accounted for by variation due to sampling. The sample size of 25 kernels was used because 25 kernels can be spaced in a Petri dish without having kernels touch. If an ear has 50% resistant kernels, a 25-kernel sample will give an estimate that is ± 20% of the actual value. Seed infection was detected by visual inspection at 6 days, but the determination of the effect of seed infection was not always obvious. For cultivar evaluation, if most kernels of an ear sample were infected with *Diploidia zeae, Fusarium graminearum,* or *Nigrospora oryzae* and there were no survivors, then the results of that ear were excluded from the cultivar average rating of *Pythium* resistance. The covered and regular tests should be useful also as part of a system to study disturbance of host cells by a soil-borne pathogen because the occurrence and sequence of certain events can be determined.

Moisture and temperature are considered primary factors of the environment important in determining the development of soil-borne pathogens. The placement of inoculum, provided that it is equidistant from kernels, would not be expected to influence mortality as it did. Geotropism is a possible explanation of increased mortality when inoculum was placed above the kernels. The mycelia of fungi are not considered geotropic, although sporocarps exhibit strong geotropism.

The difference in area of host-pathogen contact between the regular and covered tests did not account for the differential survival in the two tests. Indeed, there was slightly less contact in the covered test because of the increased aerial growth of *Pythium* on the undisturbed cultures of the regular test compared with filter-paper cultures. Distances between inoculum and kernels were approximately the same in the covered and top tests and are not considered responsible for difference in survival. The increased mortality of embryos when inoculum was placed on top was not explained by the need for the seedling to grow up through the inoculum because the embryos generally were killed before emergence in both covered and top tests.

P. debaryanum attacked root tissue and only young embryos. A distinct mesocotyl was not observed in the surface-planted kernels. The mesocotyl is attacked by *Pythium* only occasionally (2, 5, 7). Maximum resistance was exhibited by the plumule and scutellar node. If the plumule was not killed before or shortly after emergence, it became immune from further attack by *Pythium*. Some resistance was manifested by the roots, but it was less distinct than resistance in plumules. Most radicles were killed before emerging; the few radicles able to emerge seldom grew more than 3 cm before the root tip became necrotic and terminal growth ceased. An apparent manifestation of resistance was the ability to produce a profusion of secondary and seminal roots in seedlings with resistant plumules. This response of maize was noted also by Messiaen et al. (9). Greater root-rot resistance to *Pythium* is needed in dent-maize lines used as commercial seed parents (8), particularly where minimum-tillage practices are resulting in cooler soil temperatures at planting time.

It was assumed that the difference in reaction of the plumule and the radicle to *Pythium* was due to the higher level of resistance that developed in the plumule upon germination. No results were obtained to indicate a translocation of a factor for resistance from the plumule to the root. Root resistance in the absence of resistant plumules was not present, or was at such a low level that it could not be observed with present techniques. Because resistant roots were found only on surviving seedlings, it should be more efficient to search for root resistance to *Pythium* in germ plasm that has plumule resistance.

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ADDENDUM TO ANNUAL REPORT OF THE IOWA ACADEMY OF SCIENCE, 1979-80

The name of George Knaphus, Department of Botany, Iowa State University, was inadvertently omitted from the list of those who received the Distinguished Service Award on April 18, 1980. The citation was for "encouragement to generations of students, for enthusiastic support of science teaching programs, and for long-term service to the Academy."